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Source: Journal of Wildlife Diseases, 42(2) : 249-258

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-42.2.249

Journal of Wildlife Diseases, 42(2), 2006, pp. 249–258

IDENTIFICATION OF A HAEMOMYCOPLASMA SPECIES IN ANEMIC REINDEER (*RANGIFER TARANDUS*)

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ABSTRACT: During an 18-mo period (May 2002–November 2003), 10 animals in a herd of 19 reindeer (*Rangifer tarandus*) at the National Animal Disease Center (NADC) experienced episodes of anemia. Affected animals had histories of weight loss, unthriftiness, occasionally edema of dependent parts and moderate anemia characterized by microcytosis or macrocytosis, hypochromasia, schistocytosis, keratocytosis, acanthocytosis, and dacryocytosis. Numerous basophilic punctate to ring-shaped bodies, measuring less than 1.0 μm, were found on the surface of red blood cells and were often observed encircling the outer margins of the cells. Based on cytologic findings, DNA preparations from selected affected animals in the NADC herd and one animal from a private herd experiencing similar episodes of anemia were assayed by polymerase chain reaction (PCR) for the presence of hemotropic bacteria using primers targeting the 16S rRNA genes of *Mycoplasma (Eperythrozoon) suis*, *Mycoplasma (Haemobartonella) haemofelis*, *Anaplasma marginale*, *Anaplasma* spp., and *Ehrlichia* spp. Amplification products were detected from four of the affected animals using primers specific for the 16S rRNA gene of *M. haemofelis* and *Mycoplasma haemocanis*. Product from one of the animals was sequenced and internal primers were designed from the resulting sequence to perform a nested PCR assay. Samples from 10 reindeer were positive using the nested PCR reaction and products from seven animals were sequenced; BLAST searches and phylogenetic analysis were performed on the resulting sequences. Sequence data from six animals revealed homology to an organism most closely related to *Mycoplasma ovis*, *Mycoplasma wenyonii*, and *Mycoplasma haemolamae*; sequence from a single animal was most closely related to *M. haemofelis* and *M. haemocanis*. This represents the first identification of a haemomycoplasma species in reindeer. Although several animals were also infected with abomasal nematodes, the presence of this newly described haemomycoplasma may have contributed to the anemic syndrome.

Key words: Anemia, haemomycoplasma, hemoplasma, mycoplasma, *Rangifer tarandus*, reindeer.

INTRODUCTION

The genera *Haemobartonella* and *Eperythrozoon* recently have been reclassified as *Mycoplasma* based on 16S rRNA genetic analysis and morphologic similarities (Neimark et al., 2001). These wall-less bacteria are epicellular erythrocytic pathogens that have not been cultured in vitro. Haemomycoplasma organisms have been identified in a variety of domestic and wild mammals and the cytological appearance of these organisms has been associated with anemia of varying severities (Messick, 2004). Clinical signs in infected animals are similar to other anemias or hypoxic conditions and include weakness, depression, anorexia, increased capillary refill time, decreased production and rate of gain, and splenomegaly. The extravascular hemolytic anemia that occurs is often transient and recurrent as well as being regenerative, characterized by reticulocytosis, polychromasia, macrocytosis with basophilic stippling, and Howell-Jolly bodies. The clinical syndrome is usually more severe in young, splenectomized, and immunocompromised animals.

Several animals within a 19-head reindeer (*Rangifer tarandus*) herd presented with emaciation, lethargy, inappetence, tachypnea, pallor of mucous membranes, and increased capillary refill time. Two animals also had edema of dependent parts. Complete blood counts performed on index-case animals revealed moderate to severe anemia and marked alterations in erythrocyte morphology. Some animals had macrocytic, hypochromic anemias

with erythrocyte changes that included macrocytosis with hyperchromasia and basophilic stippling, marked schistocytosis, keratocytosis, acanthocytosis, and dacryocytosis. Several animals had microcytic, hypochromic anemias characterized by microcytosis with hypochromasia, schistocytosis, acanthocytosis, and dacryocytosis. Additionally, amphophilic to basophilic structures resembling organisms were observed microscopically on erythrocytes. These structures had multiple forms including single punctate, chaining punctate, clustering punctate, single bacillary, chaining bacillary, single rings, chaining rings, and clustering rings; they best resembled organisms of the old classification of *Eperythrozoon* because they occurred in ring forms and extracellularly.

During the following 18-mo period, 10 animals experienced episodes of anemia with or without exhibiting clinical signs similar to those of the index cases. Periods of anemia were usually associated with easily observed erythrocytic organisms similar to those seen in the index cases.

The objectives of this study were 1) to determine if the structures observed on erythrocytes of anemic reindeer were organisms, alterations in erythrocytic morphology, or merely artifacts and 2) to obtain genetic data on these putative organisms in order to determine if this was a newly observed organism or if it was a previously recognized erythrocyte pathogen which had crossed into a new host.

MATERIALS AND METHODS

This study was conducted from May 2002 to November 2003 at the National Animal Disease Center (NADC; Ames, Iowa, USA; 42°2′N, 93°37′W). The reindeer originated from a farm in southern Michigan and were shipped as a group to Ames in May 2002 for use as noninfected controls in tuberculosis diagnosis research. Upon arrival, the reindeer were treated for parasites with ivermectin (Ivomec, Merial Ltd., Iselin, New Jersey, USA) and vaccinated with a multivalent clostridial bacterin (Ultrabac-7, Pfizer Animal Health, Exton, Pennsylvania, USA). One reindeer (TN-1) was located in Knoxville, Tennessee (35°57′N, 83°55′W).

Blood was collected into EDTA tubes via jugular venipuncture. Smears were quickly prepared after blood collection and stained with Diff-Quik staining kit (American Scientific Products, McGaw Park, Illinois, USA) and with acridine orange (1:20,000 in citric acid buffer, pH=3.0). Complete blood counts were performed using a Beckman-Coulter Ac·Tdiff (Beckman Coulter, Inc., Miami, Florida, USA) automated hematology machine. Leukocyte differential counts were determined manually from the Diff-Quik stained blood smear. Reference intervals for this reindeer herd were previously determined for this instrument: six total-herd bleeds were taken on different dates, and the reference interval was set using the 95% interval; only data from animals that were apparently clinically normal were used ($n=111$). For PCR procedures, 100 µl of blood was spotted onto FTA cards (Whatman, Inc., Clifton, New Jersey, USA), air dried, stored at room temperature, and processed prior to use in polymerase chain reaction (PCR) assays according to manufacturer's protocol. From reindeer TN-1, total DNA was isolated from sera samples using the Qiagen DNA Blood Mini Kit according to the manufacturer's specifications (Qiagen, Inc., Valencia, California, USA).

Blood and sera were assayed by PCR using primers targeting prokaryotic 16S rRNA and the 16S rRNA gene of possible erythrocytic bacterial groups as listed in Table 1. The PCR reactions were 50 µl and contained 5 µl of purified nucleic acid from the FTA cards in Tris-EDTA and 45 µl of reaction mixture consisting of 200 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Indianapolis, Indiana, USA), 1× PCR Buffer II (Perkin Elmer, Branchburg, New Jersey, USA), 1.5 mM $MgCl_2$, 1.25 U AmpliTaq Gold polymerase (Perkin Elmer), and 0.2 µM of each upstream and downstream primer. Thermocycling was performed according to references (Table 1) with the exception of the reactions containing primers fHf5 and rHf6. Reactions containing these primers were performed using a touchdown procedure which consisted of a single step of 95 C for 10 min followed by 50 cycles of 95 C for 45 sec, a touchdown step of 67–50 C for 60 sec (the temperature was decreased by 1 C each step for the first 17 cycles), and 72 C for 120 sec.

The PCR product obtained from one animal (reindeer 107) using primers fHf5 and rHf6 was sequenced and internal primers (fHFi1 and rHfi2) were designed using PrimerQuest software (Integrated DNA Technologies, Cor-

TABLE 1. Targets and sequences of primers used in PCR assays to identify the erythrocytic organism observed in anemic reindeer.

Names	Target	Sequence
HK12[a]	Prokaryotic 16S rRNA	5′-GAGTTTGATCCTGGCTCAG-3′
HK13		5′-TACCTTGTTACGACTT-3′
fHF1[b]	Prokaryotic 16S rRNA	5′-ACGCGTCGACAGAGTTTGATCCTGGCT-3′
rHF2		5′-CGCGGATCCGCTACCTTGTTACGA-3′
fHF5[b]	*Mycoplasma haemofelis* & *Mycoplasma haemocanis*	5′-AGCAGCAGTAGGGAATCTTCCAC-3′
rHF6		5′-TGCACCACCTGTCACCTCGATAAC-3′
MSU1[c]	*Mycoplasma suis*	5′-GCATTGCCCAGTCCCCAAGGA-3′
MSU2		5′-TGCGGGGACTACGTGGGAAGG-3′
ECB[d]	*Ehrlichia* & *Anaplasma* spp.	5′-CGTATTACCGCGGCTGCTGGCA-3′
ECC		5′-AGAACGAACGCTGGCGGCAAGCC-3′
ECA[e]	*Ehrlichia* & *Anaplasma* spp.	5′-AACACATGCAAGTCGAACGGA-3′
HE3		5′-TATAGGTACCGTCATTATCTTCCCTAT-3′
fHfi1	Designed from reindeer sequence 1 IA	5′-ATCGTTTACGGTGTGGACTACTGG-3′
rHfi2		5′-AGCAGCTGCGGTAATACATAGGTC-3′

[a] Ammann et al., 1995.

[b] Messick et al., 1998.

[c] Hoelzle et al., 2003.

[d] Dawson et al., 1994.

[e] Anderson et al., 1992.

alville, Iowa, USA). Primers fHFi1 and rHfi2 were used in a nested PCR reaction with the product of reactions containing primers fHf5 and rHf6 as template. The thermocycling conditions for the reactions containing fHfi1 and rHfi2 primers were 95 C for 10 min followed by 40 cycles of 95 C for 30 sec, 60 C for 60 sec, and 72 C for 60 sec. The PCR products were visualized with ethidium bromide–stained agarose gels.

The PCR products were purified in a silica matrix (Geneclean II kit, Q-Biogene, Irvine, California, USA) prior to sequencing. Products were quantitated using the Pico Green assay for dsDNA (Molecular Probes, Eugene, Oregon, USA) and the appropriate quantity of dsDNA was labeled in both directions using Big Dye terminator chemistries and sequenced using an ABI 3100 genetic analyzer (Applied Biosystems Inc., Foster City, California, USA). Primers used for sequencing were identical to those used in the primary PCR reaction and were used at a concentration of 0.13 μM. Resulting sequences were assembled and edited using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Gene sequences for haemomycoplasma organisms as well as other Mollicutes members were obtained from GenBank. Reference sequences and the newly obtained reindeer sequences were trimmed to a similar length. Alignment of gene segments was performed with the program CLUSTALX (Thompson et al., 1997). Phylogenetic analysis of the aligned sequences was performed using the program Mega2 (Kumar et al., 2001). The phylogenetic tree was formed using the neighbor-joining method from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model with a transition/transversion ratio set at 2. Data were resampled 10,000 times, and bootstrap analysis was used for statistical assessment of the resulting node.

For transmission electron microscopy, blood samples were immediately fixed in 2.5% (v:v) glutaraldehyde in 0.1 M cacodylate buffer (pH=7.2) at a 1:10 ratio for 2 hr. Samples were centrifuged at 600 × G and rinsed three times with the cacodylate buffer, then postfixed in 1% (w:v) osmium tetroxide for 30 min and rinsed three times with cacodylate buffer. The resulting cells were dehydrated in graded concentrations of ethanol and cleared in propylene oxide. The cell pellet was infiltrated with Eponate 12 (Ted Pella, Inc., Redding, California, USA) and polymerized for 48 hr at 60 C. Thin sections were cut at 70 nm, mounted on grids, stained with uranyl acetate and Reynold's lead, and examined with a Philips 410 transmission electron microscope.

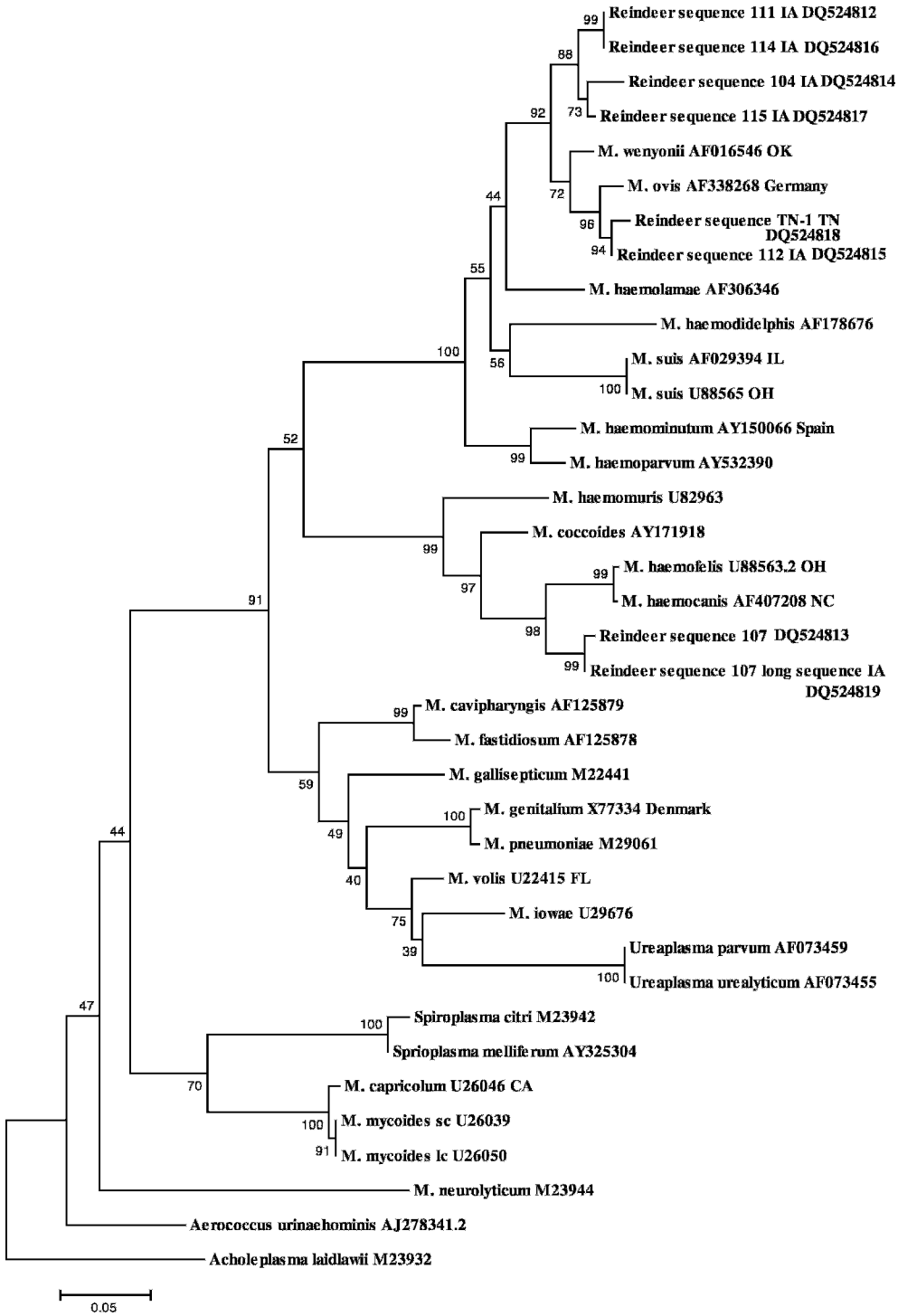

FIGURE 1. Phylogenetic tree showing positions of reindeer haemomycoplasmas relative to other haemoplasmas and other mollicutes. The phylogenetic tree was derived from 16S rRNA sequences. The GenBank accession number and origin of material (if known) are included at each axis with the species names. Bootstrap percentage values are given at each node of the tree and were obtained from 10,000 resamplings of

RESULTS

The PCR testing of blood or sera using nonspecific primers targeted to the 16S rRNA gene of prokaryotes, primers reported to amplify all members of the *Ehrlichia/Anaplasma/Cowdria* group, and primers specific for *M. suis* failed to yield product. However, primers directed against *M. haemofelis* and *M. haemocanis* (fHf5–rHf6 primer set) yielded product of the predicted size (650 base pairs [bp]) in four animals and the nested PCR assay (fHf5–rHf6 primer set followed by fHfi1–rHfi2 primer set) yielded product of the predicted size (300 bp) in nine animals from the NADC herd and one animal from a private herd in Tennessee. Nested PCR products from seven animals were sequenced. Results of sequence comparison are shown in Figure 1. Six of these sequences were highly related to the ruminant haemomycoplasmas. These six were split into the following groups: sequences from four NADC animals appear to be most closely related to *M. wenyonii* and two sequences (TN-1 and one NADC animal) appear most similar to *M. ovis*. Both sequences from reindeer 107 were more closely related to *M. haemofelis* and *M. haemocanis*.

Thirteen of the 19 reindeer in the NADC herd had evidence of a haemomycoplasma-like organism. Ten had cytologic evidence of organisms on erythrocytes (Fig. 2 and Table 2); nine had PCR evidence. Of the nine NADC animals positive by PCR, six were anemic with organisms visible on blood smears and three were asymptomatic with no organisms observed on blood smears. Polymerase chain reaction assays were not done on samples from four of the 13 affected animals and determination of the presence of a haemomycoplasma-like organism was based on cytology alone. The 10 animals with cytologic evidence of the organism were anemic (Table 2). Anemias were of multiple classifications: two animals had moderate, macrocytic, hypochromic anemia (reindeer 108 and 115); one animal had a moderate, macrocytic, normochromic anemia (reindeer 118); two animals had moderate microcytic, normochromic anemia (reindeer 112 and 100); three animals had moderate, microcytic, hypochromic anemia (reindeer 117, 107, and 105); one animal had a moderate, normocytic, normochromic anemia (reindeer 102); and one animal had a severe anemia which could not be classified (reindeer 109). The single animal from Tennessee (TN-1; 2 yr old) reportedly had episodes of transient anemia since birth and sera were positive for haemomycoplasma by the fHfi1 and rHfi2 PCR on four separate bleeding dates. Cytologic changes in erythrocytes included microcytosis, hypochromasia, schistocytosis, acanthocytosis, and dacryocytosis. Polychromasia, basophilic stippling, and Howell-Jolly bodies were also observed.

Organisms with features similar to those previously reported for haemomycoplasmas and other mycoplasmas were observed by electron microscopy (Fig. 3). Organisms were closely associated with the erythrocyte surface and separated from the erythrocyte plasma membrane by a space of 25–35 nm. Organisms measured 0.3–1.0 μm and were round or rod-shaped. The majority of organisms were round and measured approximately 0.6 μm. Organisms typically had a circumferential, peripheral electron-dense area and a central electron-lucent area that contained numerous intersecting fibrillar structures. Electron-dense punctate bodies resembling ribosomes were common throughout the organisms. Vacuoles with-

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the data set. The phylogenetic tree was constructed using the neighbor-joining method with the Kimara two-parameter model correcting for nucleotide substitutions. *Acholeplasma laidlawii* was used as an outgroup. The scale bar shows the distance equivalent to one substitution per 20 nucleotides.

in the central lucent areas were also common. Blebbing at the terminal poles was also observed in some organisms. Occasionally the terminal poles contained large vacuoles that appeared to be immediately below the plasma membrane; terminal vacuoles were associated with discontinuous cell membranes and seemed to represent partially lysed cells. The organisms lacked cell walls and contained a trilayered unit membrane, which measured 9–13 nm. The unit membrane often appeared asymmetrical with the inner layer being slightly thicker than the outer layer. Numerous intercellular organisms were observed also; these organisms tended to appear in clusters of two to 20 organisms.

DISCUSSION

This study represents the first report of a haemomycoplasma in reindeer. The presence of these haemomycoplasma organisms and the accompanying anemias were transient and recurrent within the affected reindeer. Subjectively, the organism load seemed to be correlated with the level of anemia. Although some animals also were infected with trychostrongyle abomasal nematodes, which likely contributed to their anemic states, an aggressive anthelmintic program was eventually found that eliminated the trichostrongyle abomasal nematodes; however, animals continued to experience bouts of anemia with haemomycoplasma organisms demonstrated in blood smears and by PCR after the nematodes were eliminated from the herd.

Anemia induced by extravascular hemolysis caused by a haemomycoplasma would be expected to be a strongly regenerative, macrocytic, hypochromic anemia (Gaunt, 2000). Differences in the classifications of anemia described here may be related to time of sampling and the transient presence of the organisms. Also, those animals with concurrent trichostrongyle abomasal parasites would likely have microcytic, hypochromic anemia because of chronic blood loss with iron deficiency (Harvey, 2000). Reticulocyte counts were not performed in this study; however, there was evidence on the blood smears (such as polychromatic macrocytes, Howell-Jolly bodies, and basophilic stippling) that the anemia in most reindeer with the haemomycoplasma organisms was strongly regenerative.

Phylogenetic analysis of the sequences obtained in this study suggests that there may be at least two species of haemomycoplasma organisms that are able to infect reindeer. Most of the sequences closely resembled *M. wenyonii* and *M. ovis* which both infect ruminants; however, the sequences from one animal were more closely related to the organisms *M. haemofelis* and *M. haemocanis*. The current convention in nomenclature of haemomycoplasma organisms is to name the organism with respect to the host species. However, this may lead to divergent organisms being classified as the same species. Indeed, by 16S rRNA genetic analysis, some *M. haemofelis* organisms are more closely related to other haemomycoplasma species than they are to typical *M. haemofelis* strains (Johansson et al., 1999). We propose that the newly discovered reindeer haemomycoplasma strains be given the speciation *Candidatus Mycoplasma haemotarandirangiferis*. One of the sequences obtained in this study was from a reindeer located in Tennessee that was never in close geographic contact with the other animals in this study; thus, the haemomycoplasma infection identified in this report was not a single-herd, sporadic event.

Transmission of haemomycoplasmas in other species has been shown to be accomplished by a number of blood-feeding arthropod vectors including ticks, mosquitoes, and stable flies (*Stomoxis calcitrans*) (Nikol'skii and Slipchenko, 1969; Overas, 1969; Howard, 1975; Daddow, 1980). Oral transmission has also been shown experimentally in other species (Overas, 1969) as well as transmission by blood-contaminated tools such as shears and ear taggers (Brun-Hansen et

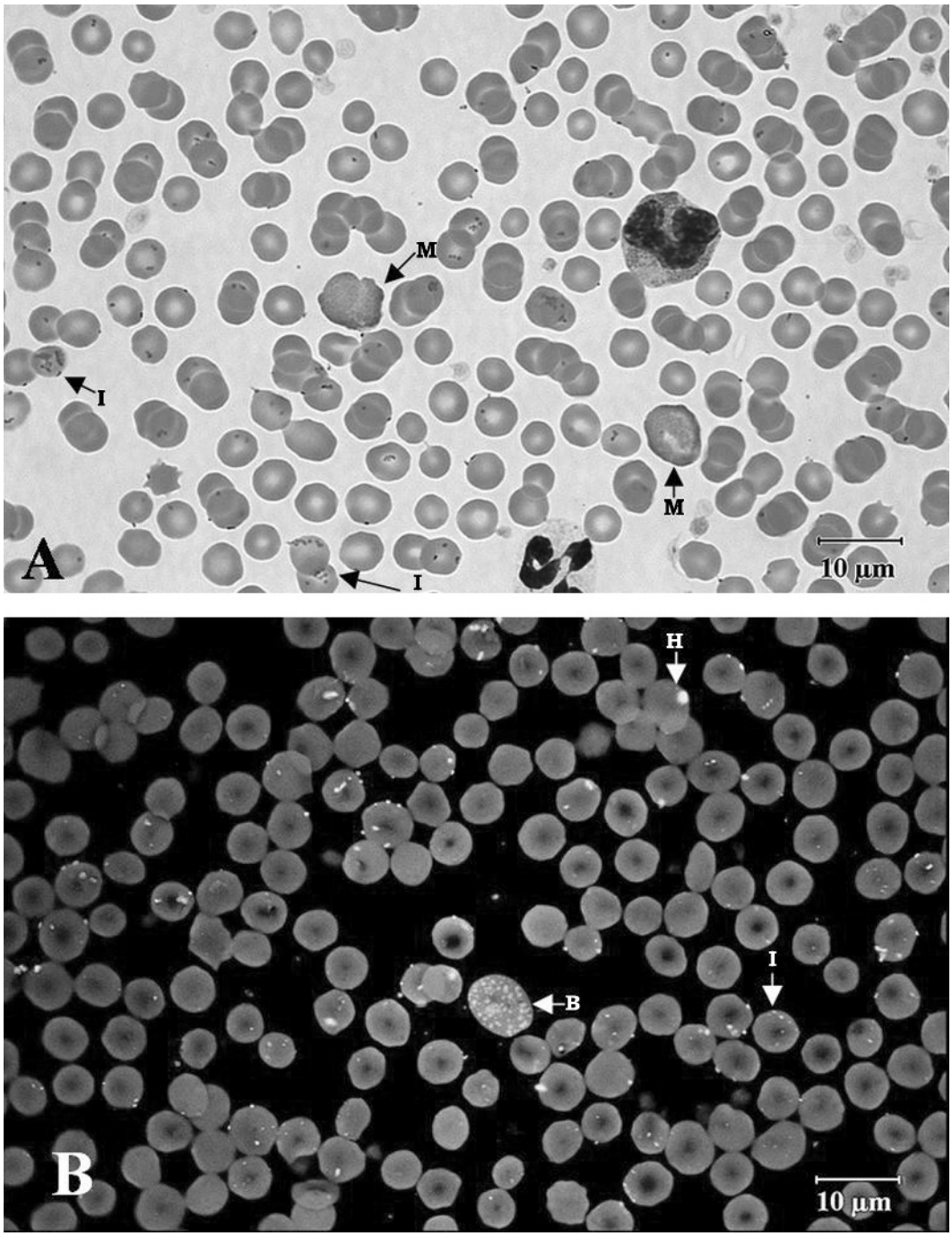

FIGURE 2. Cytologic appearance of reindeer haemomycoplasma organism. A: Diff-Quik–stained blood smears from anemic reindeer with eperythrocytic haemomycoplasma organisms. Infected cells (I) have multiple punctate, bacillary, or ring forms. Note the macrocytic cells with polychromasia (M). B: Acridine orange–stained blood smears from anemic reindeer with eperythrocytic haemomycoplasma organisms (I). Numerous intensely stained haemomycoplasma organisms can be observed on the surface of erythrocytes. Note that the Howell-Jolly bodies (H) and basophilic stippling (B) also stain with acridine orange.

TABLE 2. Complete blood counts of reindeer in which the haemomycoplasma organism was observed.

Parameter	Reference interval	Reindeer 108	Reindeer 109	Reindeer 117	Reindeer 107	Reindeer 112	Reindeer 102	Reindeer 118	Reindeer 115	Reindeer 105	Reindeer 100
RBC (M/μl)	7.8–10.4	**6.61**[c]	**2.31**	9.9	**6.61**	**5.17**	**6.8**	**5.59**	9.56	**6.27**	**6.6**
HGB (g/dl)	14.8–19.3	**9.4**	**6.7**	**11**	**3.8**	**7.7**	**11.2**	**10.5**	**14.7**	**9.6**	**12.2**
HCT (%)	37.0–52.9	42.3	−−−−[a]	**35**	**16.1**	**16.9**	**31.9**	**29.9**	54.8	**29.1**	**29.3**
MCV (fL)	47.0–51.9	**64**	−−−−	**35.4**	**24.4**	**32.6**	46.9	**53.4**	**57.3**	**46.4**	**44.4**
MCH (pg)	16.9–18.8	**14.2**	−−−−	**39.6**	**5.7**	**14.9**	16.5	18.8	**15.37**	**15.2**	18.5
MCHC (g/dl)	34.6–38.6	**22.2**	−−−−	**13.1**	**23.6**	**45.6**	35.2	35.1	**26.82**	**32.8**	**41.7**
RDW (%)	14.3–18.6	**22.7**	**54.8**	**48.6**	**37.2**	**28.1**	**28.3**	**24.6**	23.8	26.7	28.5
Platelets (K/ml)	423–1,595	412	1,985	692	663	363	439	481	528	351	2267
Mean platelet volume (fl)	4.0–8.0	10.8	19.8	4.6	9.1	12.1	++++[b]	8	5.6	10.4	11.3
WBC (No./μl)	5,100–16,400	3, 060	14,780	6,000	10,580	10,260	8,100	15,700	7,700	16,200	12,700
Neutrophils (No./μl)	2,112–9,396	2,360	13,006	4,260	4,820	7,798	3,726	12,089	5,467	9,396	7,747
Lymphocytes (No./μl)	1,700–4,550	210	1,774	1,380	5,020	2,052	3,402	2,198	1,771	1,980	3,429
Monocytes (No./μl)	134–1,480	30	0	120	360	103	162	1,099	154	297	635
Eosinophils (No./μl)	0–819	390	0	240	360	206	810	314	308	198	762
Basophils (No./μl)	0–228	0	0	0	0	103	0	0	0	99	127
Haemomycoplasma ID – cytology		+	+	+	+	+	+	+	+	+	+
Haemomycoplasma ID – PCR		ND	+	+	+	+	+	ND	+	ND	ND
Fecal flotations[d]		+	−	−	−	−	−	+	−	+	−
Parasites at necropsy		+	+	−	−	−	−	−	−	+	−

[a] −−−−− = parameter could not be resolved.

[b] ++++ = above detectable limits.

[c] Boldfaced values are outside of the reference interval.

[d] Zinc sulfate ova flotation procedure. All + samples contained trichostrongyle ova.

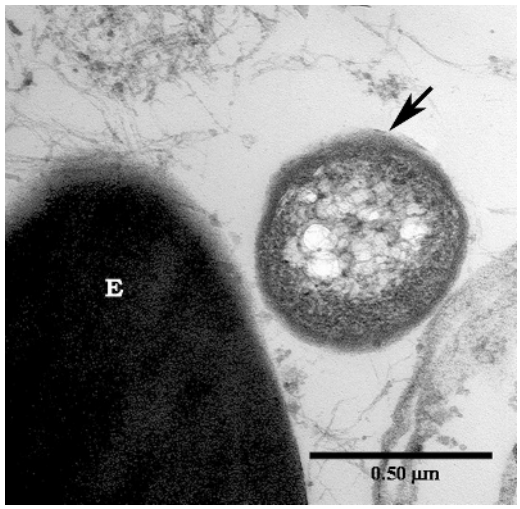

FIGURE 3. Transmission election micrograph of reindeer haemomycoplasma organism. The organism (arrow) is separated from the erythrocyte (E) plasma membrane by an apparent 25-nm space.

al., 1997) and reused needles (Mason and Statham, 1991). Because of the nonspecific nature of transmission and the close proximity of other cervids (an elk [*Cervus elaphus nelsoni*] herd was on pasture within 50 m and a white-tailed deer [*Odocoileus virginianus*] herd was on pasture within 200 m) to the infected NADC herd, future research should include investigating evidence of this organism in other deer species.

This report represents the second class of organisms to infect reindeer erythrocytes. *Babesia* spp. have also been shown to infect reindeer erythrocytes and cause acute episodes of intravascular hemolysis characterized by anorexia, hemoglobinuria, and high mortality in reindeer (Rehbinder, 1990; Holman et al., 2003; Langton et al., 2003). *Babesia odocoilei*, which is carried by the primary tick vector *Ixodes scapularis* that infects elk, white-tailed deer, and reindeer in North America, is round or pyriform measuring up to 3 µm and occurs singly, as joined pairs, or in clusters within the erythrocyte (Holman et al., 1994a, b). *Theileria cervi* is another intraerythrocytic apicomplexan organism found in multiple deer species (Chae et al., 1999b). *Theileria cervi* occurs as paired pyriform organisms within erythrocytes and can be indistinguishable from *Babesia* spp. *Theileria cervi*, which is transmitted by the primary vector *Amblyomma americanum*, is usually nonpathogenic and is most closely related to *Babesia* spp. by 16S rRNA genomic analysis (Chae et al., 1999a). Neither of these organisms resembles the size, morphology, or associated clinical syndrome of the haemomycoplasma organism reported in this paper.

The results of this study correlate the molecular diagnosis of a newly identified haemomycoplasma with an anemic syndrome and the presence of eperythrocytic organisms with typical haemomycoplasma morphology in reindeer from two states, Iowa and Tennessee. By 16S rRNA analysis, there appear to be at least two subgroups of this organism for which we propose the name *Candidatus Mycoplasma haemotarandirangiferis*. The *Candidatus* designation is appropriate, as it is reserved for newly described, incompletely characterized taxa in order to give them provisional status. Further work is required in order to determine the true pathogenic effects of this organism as well as its distribution within reindeer populations.

ACKNOWLEDGMENTS

The authors would like to thank Aileen Duit, Deborah Buffington, Rebecca Lyon, Karen Halloum, Eliza Albrecht, Travis Duit, and Rachel Renshaw for excellent technical assistance. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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Received for publication 11 January 2005.